

## Correlation of Carotenoid Production, Decreased Membrane Fluidity, and Resistance to Oleic Acid Killing in *Staphylococcus aureus* 18Z

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*Staphylococcus aureus* is susceptible to killing by host-derived fatty acids. Studies were performed to test for a correlation between carotenoid production by *S. aureus* and protection against oleic acid. Oleic acid killing of cells grown in carotenoid expression medium was determined as the dosage of oleic acid in 2 M NaCl–2 mM EDTA that would kill 20% of the cells in 60 min at 37°C (i.e., the 20% lethal dose). Compared with the wild-type strain (18Z), a carotenoid-deficient mutant strain (18Z-76) and strain 18Z grown in a medium that suppressed carotenoid production both showed increased sensitivity to oleic acid. Spontaneous revertants of strain 18Z-76 that regained the ability to produce carotenoids were as resistant to oleic acid as the wild-type strain. Oleic acid was shown by fluorescence polarization to decrease polarization values. Lower polarization values indicate a more-fluid membrane. To determine whether protection against oleic acid killing might depend on carotenoid stabilization of membranes, fluorescence polarization values were determined for strains showing different levels of carotenoid production. An indirect correlation was found between membrane fluidity and carotenoid production. We were able to conclude that there is a direct correlation between carotenoid production (i.e., cell pigmentation), cell membrane stability, and resistance to oleic acid-induced cell killing.

The in vitro bactericidal activity of fatty acids has been known for a long time (9), and it may be important in vivo in preventing, eliminating, and in some cases treating infections (8, 22, 24). Several investigators have demonstrated the presence of lipases in human milk that are able to hydrolyze milk triglycerides to fatty acids and monoglycerides and that may protect neonates from certain bacterial and enveloped viral infections (8, 22). Another way the body can prevent infection is by the production of sebum (50% lipid). Microorganisms hydrolyze the sebum triglycerides to fatty acids and monoglycerides, providing a barrier to bacterial growth on the skin (9, 24). Fatty acids and monoglycerides can also augment the immune response against infectious agents (5, 9, 12). Phospholipases present in macrophages and polymorphonuclear neutrophils hydrolyze phospholipids to produce fatty acids that kill *Staphylococcus aureus* and *Mycobacterium tuberculosis* (11, 12).

*S. aureus* can survive on the skin and occasionally cause suppurative localized lesions that result in abscess formation (19). Studies using a murine intraperitoneal abscess model have shown that one way the host controls the growth and survival of *S. aureus* within abscesses is by the production of bactericidal fatty acids and monoglycerides (4, 9). Similarly, the usefulness of the lipid mupirocin (9-hydroxy-nonanoic acid) in topically treating *S. aureus* skin infection has been reported elsewhere (17). Other hydrophobic, nonlipid agents such as miconazole and ketoconazole also demonstrate staphylocidal activity and are able to disrupt staphylococcal membranes (23).

*S. aureus* is very sensitive to the bactericidal effects of

several fatty acids (9). The bactericidal effects of fatty acids on gram-positive bacteria such as *S. aureus* are thought to result from the incorporation of these lipophilic agents into the bacterial membranes, causing increased membrane fluidity and a decrease in vital membrane-associated functions (9).

Numerous investigators have shown that bacterial carotenoids in vitro and in vivo can stabilize bacterial membranes (16, 18, 21). In particular, Rottem and Markowitz (18) demonstrated that when growth conditions were adjusted to decrease carotenoid production in *Acholeplasma laidlawii*, an increase in membrane fluidity and osmotic fragility was demonstrated (18). Over 90% of *S. aureus* strains obtained from human patients are scored as pigmented (13). Staphyloxanthin, a glycosylated carotenoid, and its pigmented precursors are lipophilic and can incorporate into the cell membrane of *S. aureus* (21). Production of carotenoids may also help *S. aureus* stabilize its cell membrane and thereby prevent potentially lethal fatty acid-induced changes in the fluidity of its membrane.

In this study, *S. aureus* strains producing different amounts of carotenoid were used to test for an association between cellular carotenoid content, membrane fluidity, and resistance to oleic acid-induced killing.

### MATERIALS AND METHODS

**Bacterial strains.** Carotenoid-producing *S. aureus* 18Z has been described previously (6, 10). Strain 18Z-76 is a nonpigmented mutant derived from strain 18Z by ethyl methane-sulfonate mutagenesis (see below). Strains 18Z-76-I2 and 18Z-76-I3 are carotenoid-producing spontaneous revertants of strain 18Z-76.

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**Media.** TYB (Trypticase yeast broth) contained trypticase peptone (BBL) at 1.7%, yeast extract (Difco) at 1%, NaCl at 0.5%, and  $K_2HPO_4$  at 0.25%. CEM (carotenoid expression medium) was TYB containing glycerol monoacetate (ICN) at 0.4% and was solidified with Bacto-Agar (Difco) at 1%. TSB was Trypticase soy broth (BBL), and TSA was Trypticase soy agar (BBL).

**Isolation of carotenoid pigment-deficient mutants and revertants.** *S. aureus* 18Z was grown at 37°C for 18 h in TYB. Mutagenesis was performed by adding 0.08 ml of ethyl methanesulfonate to 1 ml of culture and incubating the mixture at 37°C for 20 min. Eight milliliters of 0.85% NaCl was added, and the cells were pelleted by centrifugation. Cells were suspended in 10 ml of TYB, incubated at 37°C for 4 h, plated on CEM, and incubated for 48 h at 37°C and then for 24 h at room temperature. Colonies expressing less pigment than the parent strain were isolated, Gram stained, and tested for production of alpha, beta, and delta toxins and of free and bound coagulase. Only those isolates that resembled the parent, except for pigment production, were selected for further study. Revertants of strain 18Z-76 were obtained by plating  $5 \times 10^4$  CFU from a 37°C overnight TSB culture on CEM plates and recovering well-isolated, well-pigmented colonies.

**Growth conditions used to suppress or augment carotenoid production.** Growth of strain 18Z in TYB overnight at 37°C suppressed carotenoid production. To augment carotenoid production, cells were incubated on CEM plates for 24 h at 37°C and then for an additional 48 h at room temperature.

**Oleic acid killing assay.** Cells were grown in TYB or on CEM plates as described above. The cells grown on CEM plates were harvested with nylon swabs, suspended in 20 ml of 0.85% NaCl, pelleted at  $14,500 \times g$  for 15 min at 4°C, and washed once with 0.85% NaCl. The cells grown in TYB were pelleted and washed twice. After being washed, both CEM- and TYB-grown cells were resuspended in TSB containing 20% glycerol at an optical density at 600 nm ( $OD_{600}$ ) of 1.0 ( $3.3 \times 10^8$  CFU/ml) and were frozen at -60°C in 500- $\mu$ l aliquots.

Cells to be tested for sensitivity to oleic acid were thawed in an ice water bath and diluted 1:10,000 in 2 M NaCl-2 mM EDTA as previously described (5). Cells (100  $\mu$ l) were added to duplicate twofold oleic acid dilutions (100  $\mu$ l) in a 96-well sterile microtiter plate, mixed by pipetting them up and down several times, and incubated at 37°C for 1 h. Oleic acid was prepared fresh by diluting a 10-mg/ml (in 95% ethanol) stock solution 1,000-fold with 2 M NaCl-2 mM EDTA and mixing it by swirling and pipetting the mixture up and down several times. The surviving cells were enumerated by spreading 30- $\mu$ l aliquots from each well on duplicate TSA plates and incubating them at 37°C overnight. Survival was calculated (from at least four experiments per strain) by dividing the number of survivors from wells containing oleic acid by the number of survivors from wells not containing oleic acid. Percent survivor data were transformed by the method of probits (7), and a line was fitted to the points by using linear regression analysis (20). The dosage of oleic acid able to kill 20 or 50% of the organisms ( $LD_{20}$  or  $LD_{50}$ ; in micrograms per milliliters) according to the line was used only if the correlation coefficient for the line was 0.90 or greater.  $LD_{20}$ s as well as  $LD_{50}$ s were used because 50% killing of pigmented strains did not occur even at the highest oleic acid concentrations (10  $\mu$ g/ml) after 1 h of incubation at 37°C. However,  $LD_{50}$ s were also included to aid in interpreting the data.

Statistical analysis for comparing the difference in mean

$LD_{20}$ s and  $LD_{50}$ s was performed by using Scheffe's and Tukey's methods (3). Analysis probabilities less than or equal to an alpha value of 0.05 were considered statistically significant.

**Quantitation of carotenoids.** Cells were grown on CEM medium or in TYB and were harvested as described above. Cells were then suspended in 40 ml of distilled water and washed twice in distilled water as described above. Excess water was removed from the final pellets by inversion for at least 5 min followed by weighing and then freezing the pellets at -20°C overnight. After being thawed at room temperature, the carotenoids were extracted from the cells by using methanol as described previously (14). The volume of the final carotenoid-containing extract from 0.25 g of cells was placed in petroleum ether (final volume, 3 ml), and the  $A_{462}$  was obtained with a Beckman DU-70 spectrophotometer.

**Characterization of purified carotenoids extracted from pigmented and nonpigmented *S. aureus*.** The major end product of the carotenoid pathway is an orange pigment called staphyloxanthin (15, 21). A precursor of staphyloxanthin seen in adequate quantities for analysis is a yellow pigment called 4',4'-diaponeurosporenoate (14). Thin-layer chromatography with silica gel 60H plates (Analtech) and a solvent system of 13:7 petroleum ether-acetone facilitated the separation and purification of these carotenoids extracted from the strains described above. Plates were developed until the solvent front was approximately 1 cm from the top edge of the plate. Silica gel sections containing the orange and yellow spots were then scraped from the plate and placed into separate 1.5-ml centrifuge tubes. The pigments were extracted from the silica gel by suspending the gel in acetone, pelleting it by centrifugation ( $14,000 \times g$ , 2 min), and removing the carotenoid-containing supernatants.

Absorbance maxima for wavelengths between 550 and 350 nm in petroleum ether, methanol, and acetone were obtained spectrophotometrically. The absorbance maxima for staphyloxanthin (an orange pigment) in the above solvents have been determined and are 462 nm for petroleum ether, 460 nm for methanol and 460 nm for acetone. The absorbance maxima for 4',4'-diaponeurosporenoate (a yellow pigment) in the same solvents have also been determined and are 432, 455, and 483 nm for petroleum ether, and 475 nm for methanol, and 455 nm for acetone (14).

Treatment of staphyloxanthin with 5% KOH in acetone has been shown to convert the ester (staphyloxanthin) to the free acid (4',4'-diaponeurosporenoate) (14). The orange pigments from strains 18Z and 18Z-76 were treated with alkaline acetone as described elsewhere (14), and the absorbance maxima of the products were determined as described above.

Lithium aluminum hydride in dry diethyl ether is able to reduce the free acid (4',4'-diaponeurosporenoate) to an alcohol (4',4'-diaponeurosporenol) (14). The spectrum of the resulting alcohol (413, 435, and 465 nm in petroleum ether) is quite different from that of the free acid (432, 455, and 483 nm in petroleum ether) and thus is helpful in identifying the carotenoid. The yellow pigments from strains 18Z and 18Z-76 were treated with lithium aluminum hydride as described elsewhere (14), and the absorbance maxima of the products were determined as described above. The yellow pigment that resulted from treating the orange pigment from strains 18Z and 18Z-76 with 5% KOH-acetone was also treated with lithium aluminum hydride, and then the absorbance maxima were determined.

**Determination of fluorescence polarization values.** Polariza-

TABLE 1. Effect of carotenoid presence on membrane fluidity and survival after oleic acid treatment of *S. aureus*

Strain	Amt of carotenoid <sup>a</sup> ( $\pm$ SD)	Polarization value <sup>b</sup> ( $\pm$ SD)	LD <sub>50</sub> ( $\mu$ g/ml) <sup>c</sup> ( $\pm$ SD)	LD <sub>50</sub> ( $\mu$ g/ml) <sup>c</sup> ( $\pm$ SD)
18Z-76	0.292 (0.100)	0.289 (0.004)	0.39 (0.06)	1.57 (0.14)
18Z-TYB <sup>d</sup>	0.720 (0.120)	0.294 (0.002)	0.61 (0.31)	1.55 (0.78)
18Z	8.23 (4.13)	0.346 (0.007)	2.42 (0.81)	>10.0
18Z-76-I2	10.8 (3.61)	0.350 (0.011)	3.04 (0.57)	>10.0
18Z-76-I3	9.60 (2.45)	0.350 (0.007)	3.00 (0.84)	>10.0

<sup>a</sup> A<sub>462</sub>/0.25 g of cells. Values are the means of three different experiments for cells grown on CEM or TYB.

<sup>b</sup> Lower values indicate a more-fluid membrane. Values (37°C) are the means of four experiments.

<sup>c</sup> Means of four experiments.

<sup>d</sup> *S. aureus* 18Z grown in TYB medium to inhibit carotenoid production.

tion values for various *S. aureus* strains were obtained by methods analogous to those previously described for mammalian cells (1, 2). Briefly, the cells grown on CEM plates or in TYB were suspended in 0.85% NaCl containing 2  $\mu$ M 1,6-diphenyl-1,3,5-hexatriene (DPH), diluted in DPH-0.85% NaCl to an OD<sub>600</sub> of 0.300 ( $\pm$ 0.005), and then incubated at 37°C for at least 30 min. Polarization values were obtained in a SLM Aminco 8000C fluorimeter (excitation, 365 nm; emission, 425 nm). Data were acquired at various temperatures, and polarization values were calculated by using software provided by SLM.

Unincorporated DPH was removed from cells before treatment with oleic acid by pelleting the cells in a centrifuge as described above and suspending them in 0.85% NaCl. The cells were pelleted again and then suspended in 0.85% NaCl. The cells were pelleted again and then suspended in 0.85% NaCl to an OD<sub>600</sub> of 0.300 ( $\pm$ 0.005). Various amounts of a stock solution oleic acid (10 mg/ml in 95% ethanol) were added to the washed cells before they were incubated for 20 min at 37°C and polarization values were determined.

## RESULTS

**Frequency of isolating nonpigmented mutants and reversion of a nonpigmented mutant to wild type.** *S. aureus* 18Z was treated with ethyl methanesulfonate, and 115 of 25,000 CFU were isolated on CEM plates that produced either no pigment or significantly less pigment. The mutation rate was  $4.6 \times 10^{-3}$ .

The reversion frequency for a nonpigmented mutant, *S. aureus* 18Z-76, was determined by placing 50,000 CFU on CEM plates and incubating the plates as indicated in Materials and Methods. Of every 200 CFU of 18Z-76, 1 CFU spontaneously reverted to wild-type production of pigment.

**Determination of carotenoid contents of pigmented and nonpigmented strains of *S. aureus*.** The carotenoid contents of the *S. aureus* strains were determined as described in Materials and Methods. Carotenoid production by *S. aureus* 18Z was higher on CEM plates than in TSB or on TSA (data not shown). Quantitation of carotenoid content in these strains demonstrated that the parent strain (*S. aureus* 18Z) and the two spontaneous revertant strains of mutant *S. aureus* 18Z-76 (I2 and I3) produced much more pigment than the mutant strain produced on CEM plates (Table 1). Growth of *S. aureus* 18Z in TYB medium was able to suppress carotenoid expression (Table 1).

Pigments from the *S. aureus* strains were partially purified by thin-layer chromatography, treated with various reagents, and identified by their characteristic absorbance maxima at 550 to 350 nm of light (14). Even though strains 18Z-76 and 18Z grown in TYB produced less pigment than

the pigmented strains, they produced orange and yellow pigments that had the same mobility in thin-layer chromatography as pigments from the pigmented strains of *S. aureus*. The absorbance maxima of the orange pigments from strains 18Z and 18Z-76 in various solvents and when treated with alkaline acetone followed by lithium aluminum hydride demonstrated that the pigments were similar to staphyloxanthin (data not shown). The absorbance maxima of the yellow pigments from strains 18Z and 18Z-76 in various solvents and when treated with lithium aluminum hydride demonstrated that they were similar to 4',4-diaponeurosporenoate (data not shown).

**Sensitivity of cells to oleic acid killing.** More than 45 min of incubation at 37°C with oleic acid was required to obtain substantial staphylocidal killing (Fig. 1). We selected an oleic acid treatment time of 60 min at 37°C to determine the LD<sub>20</sub> and LD<sub>50</sub> of each strain, because a 60-min treatment resulted in substantial killing without excessive drying of the samples.

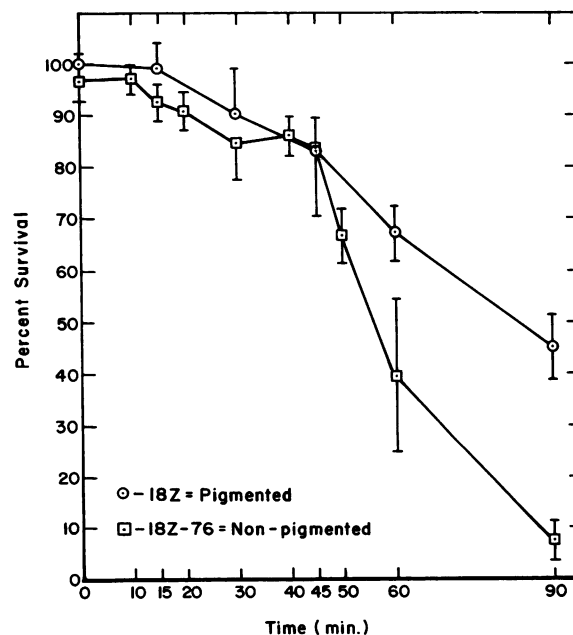


FIG. 1. Survival of *S. aureus* 18Z (pigmented) and 18Z-76 (non-pigmented) in 5  $\mu$ g of oleic acid per ml versus time. Cells ( $3.3 \times 10^3$  CFU/ml) from CEM plates were incubated in 5  $\mu$ g of oleic acid per ml for various times at 37°C and then plated on TSA. Organisms not treated with oleic acid were incubated for the same times and served as controls. Data are means from three different experiments.

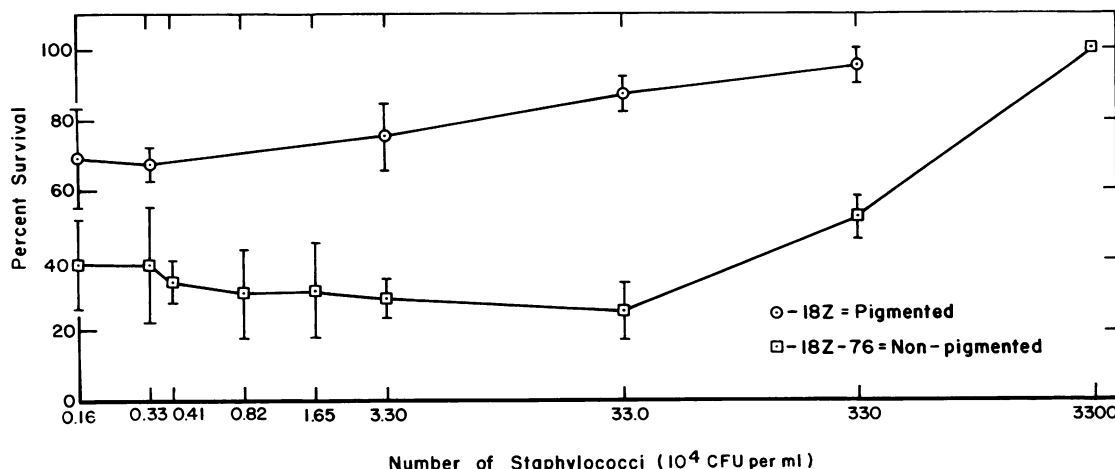


FIG. 2. Survival of increasing numbers of *S. aureus* 18Z (pigmented) and 18Z-76 (nonpigmented) in 5  $\mu$ g of oleic acid per ml. Cells from CEM plates were incubated in oleic acid for 1 h at 37°C. Data are means from three different experiments.

The effect of bacterial concentration on oleic acid killing was also determined. Incubation of a constant amount of oleic acid (5  $\mu$ g/ml) with increasing numbers of cells for 60 min at 37°C revealed that cell killing was not affected by the number of cells treated with oleic acid when the number of cells was less than  $3.3 \times 10^5$  CFU/ml for nonpigmented 18Z-76 or less than  $3.3 \times 10^4$  CFU/ml for pigmented 18Z (Fig. 2).

The LD<sub>20</sub>s and LD<sub>50</sub>s for *S. aureus* strains were also determined as described in Materials and Methods, and they demonstrated that strains producing relatively little carotenoid either via mutation or by suppression in TYB were more sensitive to oleic acid than the carotenoid-producing parental and revertant strains (Table 1). Significant statistical differences were observed when the mean LD<sub>20</sub>s or LD<sub>50</sub>s of pigmented and nonpigmented *S. aureus* strains were compared by using either the Scheffe or the Tukey test (alpha value = 0.05).

**Determination of polarization values for pigmented and nonpigmented strains of *S. aureus*.** Several investigators have shown that polarized fluorimetry can measure membrane fluidity (1, 2). Higher polarization values indicate that the membrane is less fluid. Polarization values were obtained by using DPH-treated pigmented and nonpigmented *S. aureus* strains. Mean polarization values for pigmented *S. aureus* strains were higher than those for the nonpigmented mutant and the TYB-suppressed parental strain (Table 1).

**Effects of temperature and oleic acid on polarization values of pigmented and nonpigmented *S. aureus* strains.** Polarization values were obtained at various temperatures for strains 18Z (pigmented) and 18Z-76 (nonpigmented). Polarization values changed more rapidly per degree change in temperature for the nonpigmented strain than for the pigmented strain (Fig. 3). Increasing the temperature increased the membrane fluidity much more for the nonpigmented strain than for the pigmented strain.

The addition of various amounts of oleic acid to pigmented and nonpigmented strains also affected their polarization values. Oleic acid increased the membrane fluidity of both strains in a similar fashion, but the polarization values for the pigmented strain were always higher than those for the nonpigmented strain (i.e., the nonpigmented strain always

had a more-fluid membrane than the pigmented strain at the same oleic acid concentration) (Fig. 4).

## DISCUSSION

Studies by Dye and Kapral demonstrated that strains of *S. aureus* that are resistant in vitro to killing by bactericidal lipids are also better able to survive in murine intraperitoneal abscesses. Likewise, *S. aureus* strains that are quite sensitive in vitro to killing by bactericidal lipids are poor at surviving in murine intraperitoneal abscesses (5).

Our study has demonstrated a correlation between pigment production and resistance of *S. aureus* to the bactericidal effects of oleic acid. Oleic acid was selected as the representative lipid because it is the most abundant of the bactericidal fatty acids found in *S. aureus* 18Z-induced abscesses (oleic acid, 43%; linoleic acid, 3%) (5) and because other studies have shown it to be very effective in killing gram-positive bacteria (5, 9). The pigmented strain of *S.*

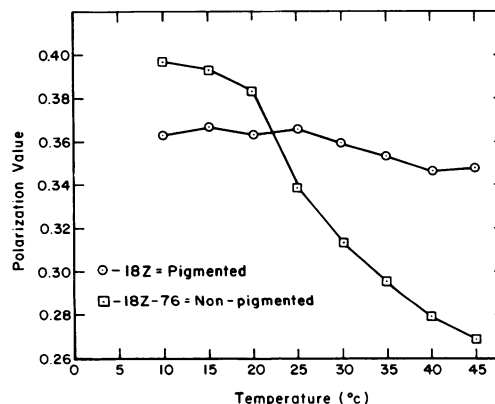


FIG. 3. Polarization values of *S. aureus* 18Z (pigmented) and 18Z-76 (nonpigmented) at various temperatures. Cells were suspended at an OD<sub>600</sub> of 0.300 in 0.85% NaCl-DPH at various temperatures, and polarization values were calculated from data obtained from the fluorimeter.

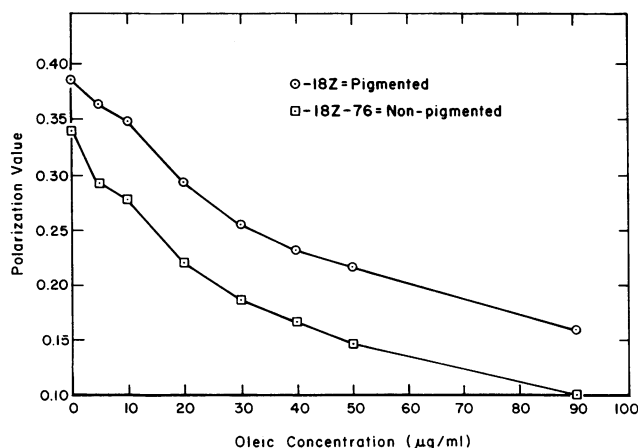


FIG. 4. Polarization values of *S. aureus* 18Z (pigmented) and 18Z-76 (nonpigmented) after various concentrations of oleic acid had been added. Lower polarization values indicate a more-fluid membrane structure.

*aureus* was less sensitive than the nonpigmented mutant to the destructive effects of treatment with oleic acid.

The nonpigmented mutant we chose for this study had a very high spontaneous reversion frequency. Since the pigmentation marker is not a selectable marker, a mutant with a high reversion frequency was required to obtain revertants. Even with a very high reversion rate, there were still differences between the mutant and wild-type strains' abilities to survive oleic acid treatment. In addition, pigment-producing spontaneous revertants of the nonpigmented mutant regained resistance to in vitro killing by oleic acid. Since over 90% of the *S. aureus* strains isolated from human infections are pigmented (13), pigmentation may be another mechanism the bacterium uses for survival in vivo.

Fatty acids appear to kill bacteria directly by inhibiting the enzymes necessary for energy production and indirectly by structurally changing the membrane itself (9). A decrease in polarization values after treatment of *S. aureus* with oleic acid indicated an increase in membrane fluidity (Fig. 4). This increase in membrane fluidity could be detected by as early as 30 s after treatment (data not shown). However, significant killing of oleic acid-treated *S. aureus* did not occur for at least 45 min (Fig. 1). These experiments suggest that oleic acid-induced killing of *S. aureus* involves multiple steps, of which the first is increasing the fluidity of the membrane.

In agreement with studies showing that carotenoids are important in decreasing the membrane fluidity of bacteria (18), our study also demonstrates that there is an indirect correlation between the amount of carotenoid present and the membrane fluidity of staphylococcal membranes (Table 1).

In conclusion, it appears that carotenoid production decreases membrane fluidity in *S. aureus* and that this decrease offers protection from oleic acid, which seems to kill *S. aureus* by initially increasing the membrane fluidity. Evidence for this conclusion is demonstrated by the decrease in sensitivity of pigment-producing strains of *S. aureus* to oleic acid treatment (Table 1) and by the fact that pigmented strains of *S. aureus* possessed less-fluid membranes to begin with and required more oleic acid to increase the membrane fluidity to the level seen for nonpigmented strains of *S. aureus* (Fig. 4).

In the future, animal models will be used to determine

whether pigment production is important in protecting *S. aureus* in vivo. Pigmented and isogenic nonpigmented mutants will be used for these studies.

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